APPENDIX E

[1]University of Virginia, Charlottesville, Virginia, USA and [2]Instituto de Biotechnolgia, UNAM, Cuernavaca, Mexico.
E-mail: jwf8x@virginia.edu

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Epidermal Transglutaminase Deposits in Perilesional and Uninvolved Skin in Patients with Dermatitis Herpetiformis

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TO THE EDITOR

Dermatitis herpetiformis (DH) is a disease precipitated by ingestion of gluten and characterized by IgA deposits in the dermal papillae (Nicolas *et al.*, 2003). Like celiac disease, DH is one presentation of gluten-sensitive enteropathy (Zone, 2005). In susceptible individuals, ingestion of gluten leads to elaboration of antibodies against gliadin and transglutaminase 2 (TG2) (Oxentenko and Murray, 2003; Alaedini and Green, 2005; Zone, 2005). TG2, one of nine homologous transglutaminases, is an autoantigen in celiac disease (Dieterich *et al.*, 1997; Lorand and Graham, 2003). Serum antibodies against TG2 can be sensitive and specific for DH as well as celiac disease (Dieterich *et al.*, 1999).

Sardy *et al.* (2002) hypothesized that an autoantigen related to TG2 could explain the presentation of DH and reported that epidermal transglutaminase (TG3) deposits with IgA in perilesional biopsies in patients with DH. IgA deposits can occur in regions that are not clinically involved (Fry *et al.*, 1978; Zone *et al.*, 1996). To confirm the involvement of TG3 in DH and to determine whether TG3 deposits are restricted to perilesional skin, we have raised a new goat antibody against human TG3.

In a series of nine DH patients with active disease, we compared direct immunofluorescence (DIF) for IgA and TG3 in perilesional and uninvolved skin. We also compared serology for TG2 and TG3 and results from DIF for IgA and TG3 in the context of a gluten-free diet (GFD). The protocol was approved by our Institutional Review Board, patients consented in writing, and we adhered to the Declaration of Helsinki Principles. All patients had a diagnosis of DH established by skin biopsy showing granular IgA and had active disease. Adherence to GFD was reported by patients as strict or poor.

Serum was analyzed for IgA antibodies against TG2, TG3, and endomysium endomysial antibody (EMA). Semiquantitative detection of anti-TG2 and -TG3 were performed using ELISA (INOVA Diagnostics, San Diego, CA, and Immunodiagnostik AG, Bensheim, Germany). EMA were detected using indirect immunofluorescence on monkey esophagus (University of California at Davis) (Unsworth, 1996). Patient age, sex, family history, adherence to GFD, dapsone use, and serologies for antibodies against TG2, TG3, and endomysium are represented in Table 1. Five patients reported strict adherence to a GFD. Among patients adhering to a strict GFD, four of five (80%) were negative by IgA ELISA for anti-TG2 antibodies and EMA. All four patients reporting poor GFD adherence had anti-TG2 antibodies and two of four were positive by EMA. All five patients in the strict GFD group were seronegative for anti-TG3 antibodies and all four patients in the poor GFD group were positive. The two patients with negative EMA had the lowest values for anti-TG2.

Abbreviations: DIF, direct immunofluorescence; DH, dermatitis herpetiformis; EMA, endomysial antibody; GFD, gluten-free diet; TG2, transglutaminase 2 or tissue transglutaminase; TG3, transglutaminase 3 or epidermal transglutaminase

Polyclonal TG3 antibody

Purified TG3 expressed from a baculovirus system was used to produce polyclonal antibodies in goats (Ahvazi and Steinert, 2003). Purified TG3 was a kind gift from Dr Bijan Ahazi. To confirm reactivity and specificity, Western blots were performed using the baculovirus-produced TG3 as well as separately cloned TG2 and TG3. Complementary DNAs for TG3 and TG2 were isolated by RT-PCR from human keratinocytes using primers published by Sardy *et al.*, and were sequence confirmed. Complementary DNA was cloned in the Invitrogen pSecTag2/Hygro B vector, lysates were purified using a Ni^{2+} column (Invitrogen, Carlsbad, CA, USA), electrophoresed, and transferred to nitrocellulose blots. IgG was prepared using a Protein G column. Western blots confirmed a high titer against both TG3 clones. Specificity was demonstrated by lack of reactivity against non-transfected human embryonic kidney lysate, recombinant TG2 (Figure 1), and serum and secretory (colostral) IgA (not shown). Further specificity was established by lack of reactivity on IIF when reacted with skin from three patients with linear IgA bullous dermatosis (Figure 2e and f), two with Henoch–

Table 1. Patient characteristics and serology

Patient	Sex	Age	FHx	GFD	[1]Dapsone	Anti TG2-IgA units	IgA-EMA, titer	IgA anti-TG3
DH1	M	61	No	Strict	No	6	0	6
DH2	F	28	No	Strict	No	6	0	5
DH3	F	54	Yes	Strict	100 mg	7	0	5
DH4	M	29	No	Strict	200 mg	11	0	3
DH5	M	72	Yes	Strict	25 mg	67	1:80	14
DH6	M	82	Yes	Poor	No	25	0	37
DH7	M	61	No	Poor	25 mg	36	0	20
DH8	F	54	Yes	Poor	200 mg	122	1:1280	20
DH9	M	66	No	Poor	No	>192	1:1280	42

EMA, endomysial antibody; F, female; GFD, gluten-free diet; M, male.
[1]All patients taking dapsone stopped medication for 48–72 hours before biopsy and evaluation.

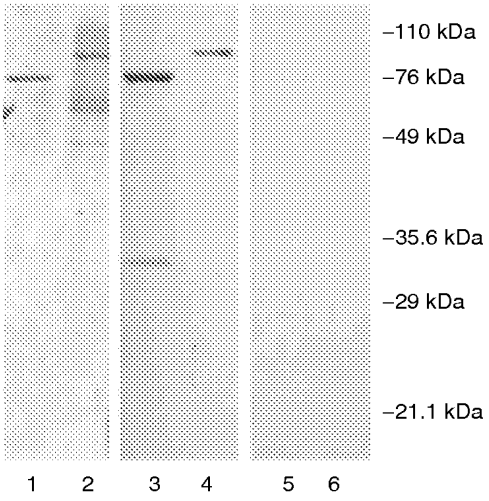

Figure 1. Coomassie-stained polyacrylamide gel (lanes 1–2) and Western Immunoblot (lanes 3–6) demonstrating reactivity of goat antisera with TG3. Baculovirus produced TG3 from the NIH and TG3 expressed in human embryonic kidney cells and eluted from a denaturing column were used as substrate and 300 ng loaded as follows: (1) NIH TG3, (2) human embryonic kidney TG3, (3) NIH TG3, (4) human embryonic kidney TG3, (5) NIH TG3, and (6) human embryonic kidney TG3. Lanes 3 and 4 are stained with an IgG-fraction of goat anti-TG3 at 1:300 and lanes 5 and 6 are stained with preimmune goat sera (IgG-fraction).

Shönlein purpura and human tonsil (not shown).

DIF

Sites of biopsy were chosen as non-erythematous skin adjacent to lesions (perilesional) and uninvolved skin 5 cm away. Four sections of each biopsy specimen were incubated with sheep anti-IgA FITC (1:40), goat IgG anti-TG3 (1:50), or preimmune goat IgG (1:50) for 30–60 minutes and washed. Anti-TG3 and preimmune control slides were incubated with FITC swine anti-goat IgG antibody (1:100) for 30 minutes before washing again. A single-blinded observer (J.J.Z.) graded all specimens by a semiquantitative scale (0–3+) (Zone *et al.*, 1996). Table 2 shows semiquantitative immunofluorescence values assigned to specimens from each of the nine patients. Typical staining patterns are shown in Figure 1a–d. All patients had granular deposits of IgA in the dermal papillae. Eight of nine had overlapping deposits of TG3. Only one specimen revealed IgA and TG3 deposition in vessel walls in the papillary dermis. TG3 showed staining of variable intensity of the upper layers of the epidermis, shown in Figure 1d. No IgA or TG3were seen in any specimen within the stratum basale.

IgA DIF was more intense than TG3 in 13 of 18 specimens and equivalent in the remaining five. IgA and TG3 DIF were greater with poor GFD adherence. The only patient negative for TG3 was on a strict GFD and had sparse IgA in perilesional skin only. All five specimens from control subjects were negative for IgA and TG3 immunofluorescence, and the three LABD and two HSP patients were positive for IgA but negative for TG3.

Patients with DH have TG3 in the papillary dermis overlapping with the deposits of IgA. Using a new antibody raised in goat with specificity confirmed using a separately cloned recombinant protein, we have confirmed findings of Sardy *et al.* suggesting TG3 is the autoantigen of DH. We also found TG3 deposits in uninvolved skin at least 5 cm away from lesions suggesting that factors beyond these complexes are necessary for DH eruptions.

IgA deposits were seen in all specimens where TG3 was found, suggesting TG3 is bound by autoantibodies as the mechanism of deposition. TG3 was not been found in the dermis in the absence of IgA. The intensity of IgA by DIF roughly correlated with the intensity of staining for TG3.

As TG3 is strongly expressed in the upper epidermis (Sardy *et al.*, 2002), it is reasonable to suspect that in regions of trauma, it may be released from the epidermal keratinocytes and diffuse through the basement membrane. Circulating autoantibodies could then bind this antigen and form characteristic deposits. An alternate explanation involves circulating immune complexes that deposit at the dermoepidermal junction (Sardy *et al.*, 2002; Preisz *et al.*, 2005), although we saw only one such involved vessel. Prior reports demonstrated IgA nephropathy in DH patients (Helin *et al.*, 1983; Reunala *et al.*, 1983). Although circulating complexes could cause this pattern, such deposits could also result from a reaction against kidney TG3 (Sardy *et al.*, 2002).

Reported sensitivity of anti-TG2 ELISA and EMA for DH has varied (Chor-

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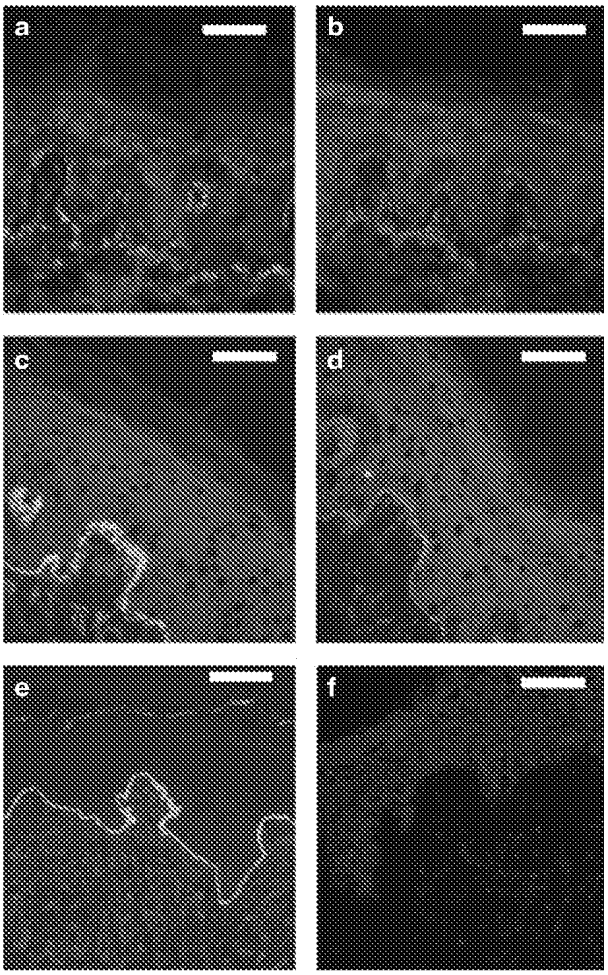

Figure 2. DIF in for IgA and TG3. (**a–d**) Specimens are from patient DH5. (**a**) Uninvolved skin showing 2+ DIF for IgA. (**b**) Uninvolved skin showing 1+ DIF for TG3. (**c**) Perilesional skin showing 3+ DIF by IgA. (**d**) Perilesional skin showing 2+ granular DIF by TG3 and some staining of the stratum corneum. (**e**, **f**) Specimens from a single biopsy of an LABD patient, although not adjacent cuts. (**e**) DIF for IgA showing a linear pattern. (**f**) No reaction with anti-TG3. Bar = 0.05 mm.

Table 2. Semiquantitative DIF for perilesional and uninvolved skin biopsies

	Perilesional			Uninvolved		
Patient	**IgA**	**Tg3**	**Neg**	**IgA**	**Tg3**	**Neg**
DH1	2+DP	1+DP	0	2+DP	1+DP	0
DH2	1+DP	0	0	0	0	0
DH3	2+DP, DD	1+DP, DD	0	2+DP, DD	1+DD	0
DH4	1+DP	1+DP	0	1+BM, VW	1+BM, VW	0
DH5	3+DP continuous	2+DP continuous	0	2+DP, DD	1+DP, DD	0
DH6	3+DP continuous	2+DP	0	2+DP continuous	1+DP	0
DH7	2+DP	1+DP	0	1+DP	1+DP	0
DH8	2+DP	1+DP	0	1+DP	1+DP	0
DH9	3+DP continuous	2+DP continuous	0	3+DP	2+DP	0

BM, basement membrane; DD, deep dermis, DIF, direct immunofluorescence; DP, dermal papillae, VW, vessel walls.

zelski *et al.*, 1986; Reunala *et al.*, 1987; Volta *et al.*, 1992; Koop *et al.*, 2000; Sardy *et al.*, 2000). Studies correlate positive serology with more severe intestinal damage (Volta *et al.*, 1992) and seronegativity with adherence to GFD (Peters and McEvoy, 1989; Volta *et al.*, 1992). Serology by IgA ELISA for anti-TG3 and TG2 antibodies correlated with adherence to GFD. Interestingly, EMA did not correlate tightly with TG3 or TG2 (Table 1). Mean DIF was greater for IgA and TG3 in patients seropositive for anti-TG2 and TG3 antibodies and reporting poor adherence to GFD, but a definitive relationship between serology and immune complex deposition cannot be established from this small patient series.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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Matthew R. Donaldson[1], John J. Zone[1], Linda A. Schmidt[1,2], Ted B. Taylor[1], Susan L. Neuhausen[3], Christopher M. Hull[1], Laurence J. Meyer[1,2]

[1]Department of Dermatology, University of Utah, Utah, USA; [2]Veterans Administration Medical Center, Salt Lake City, Utah, USA and [3]Division of Epidemiology, Department of Medicine, University of California, Irvine, California, USA
E-mail: meyer@derm.med.utah.edu

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Regulation of IL-13 receptors in Human Keratinocytes

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TO THE EDITOR

The pathophysiological features of chronic inflammatory skin diseases such as allergic contact dermatitis and atopic dermatitis are characterized by the infiltration of a large number of inflammatory cells, particularly Th2 lymphocytes (producing IL-13 and/or IL-4 in acute lesions) and Th1 lymphocytes (producing IFN-γ in chronic lesions) (Leung *et al.*, 2004). The presence of IL-13-positive cells in acute lesions of atopic dermatitis (Hamid *et al.*, 1996) and the importance of IL-13, independent of IL-4 in generation of Th2 immune response in skin compartment has been described.

In human primary keratinocytes (KCs), IL-13 interacts with two different receptors: one is the IL-4 type II receptor complex (IL-4Rα and IL-13Rα1) and the other is high-affinity IL-13Rα2. IL-13Rα1 is expressed on nearly all cells including KCs except on T cells and signals via JAK/signal transducer and activator of transcription (STAT) pathways (Hershey, 2003). IL-13Rα2 interacts with IL-13 but not with IL-4 and has been suggested to function as a decoy receptor because the cytoplasmic region of IL-13Rα2 does not have a signaling motif or JAK/STAT binding sequence (Kawakami *et al.*, 2001). However, it has recently been suggested that IL-13Rα2 might also be involved in IL-13 signaling via AP-1 (Fichtner-Feigl *et al.*, 2006). Nevertheless, very little is known about the expression and mechanism regulating the expression of IL-13Rα1 and IL-13Rα2 on KCs. With this study, we examine how cytokines involved in inflammatory skin diseases can influence the IL-13 responses of KCs by modulating the expression of IL-13Rα1 and IL-13Rα2.

To get insight into the cytokine regulation of IL-13Rα1 expression on KCs, we analyzed surface expression by flow cytometry using specific mAbs. In all experiments, there was a surface expression of IL-13Rα1 detectable on KCs (Figure 1a). We used several cytokines such as transforming growth factor (TGF)-β1 (0.1–10 ng/ml) and IL-1β, TNF-α, IFN-γ, IL-4, GM-CSF, and IL-13 at 10 ng/ml to demonstrate the modulation of IL-13Rα1 on KCs. As depicted in Figure 1a, TGF-β1 (1 ng/ml) upregulated the expression of IL-13Rα1 significantly (mean fluorescence intensity $\pm$ SEM in unstimulated; 10.93 ± 1.7 and TGF-β1; 14.72 ± 2.4, $P = 0.002$, paired t-test, $n = 7$). TGF-β1 at 0.1 and 10 ng/ml concentrations also induced IL-13Rα1 on KCs significantly (data not shown). However, IL-1β, TNF-α, GM-CSF, IL-4, and IFN-γ did not show significant effects on the modulation of IL-13Rα1 expression (data not shown). Previously, IFN-γ (100 ng/ml) and IL-13 (100 ng/ml) have been described to upregulate IL-13Rα1 at messenger RNA level (Wongpiyabovorn *et al.*, 2003). We could not observe modulation of IL-

Abbreviations: KC, human primary keratinocyte; STAT, signal transducer and activator of transcription; TGF, transforming growth factor